Asian Herpetological Research 2014, 5(2): 104–112
DOI: 10.3724/SP.J.1245.2014.00104

Characterisation and Expression Analysis of *Sox9* in the Multiocellated Racerunner, *Eremias multiocellata*

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Abstract *Sox9* is an important member of *Sox* family which is involved in a variety of developmental processes including sex determination and gonadal differentiation. The cDNA of *Sox9* from multiocellated racerunner *E. multiocellata* was cloned using reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). The sequence contains a 1497 bp open reading frame, which encodes a 498 amino acid protein with a predicted molecular weight of 55.45 kDa. *EmSox9* displays high similarity to those of reptiles, and shows an overall amino acid identity of >82%. We also investigated the tissue-specific expression of *EmSox9* mRNA by real-time quantitative PCR. *Sox9* mRNA is present in brain, heart, liver, kidney, gonads and muscle tissues of adult *E. multiocellata*, with the highest expression in brain and testis. The results indicate that *Sox9* may play important roles in some tissues during *E. multiocellata* neural and gonadal development.

Keywords *Sox9*, HMG-box, RACE, *Eremias multiocellata*, tissue specificity

1. Introduction

In most mammals, male sex determination is initiated by the *Sry*, which is regarded as the mammalian Y-linked testis-determining gene (Gubbay *et al.*, 1990). *Sry* belongs to the *Sox* gene family, a group of related transcription factors characterized by the presence of a conserved HMG-box DNA binding domain (Bowles *et al.*, 2000). Based on sequence homology in their HMG-box, the genes of *Sox* family found in a wide variety of species are divided into ten groups (A–J). It has been demonstrated that *Sox* proteins can bind DNA in a sequence-specific manner to play an essential role in cell fate decisions in numerous developmental processes (Bowles *et al.*, 2000; Schepers *et al.*, 2002).

In addition to *Sry*, the group E *Sox* genes including *Sox8*, *Sox9* and *Sox10* are expressed during mammalian testis development (Barrionuevo and Scherer, 2010). Here we concentrate on *Sox9* which is a pivotal candidate gene associated with sex determination as it appears to be the direct target of Sry in therian mammals (Valenzuela, 2010). Mutations that alter the expression or coding sequence of *Sox9* have been found in bone dysmorphology syndrome campomelic dysplasia (CD) and abnormal gender phenotypes (Bishop *et al.*, 2000). These biological functions of *Sox9* demonstrate that this gene is involved not only in skeletal development but also in sex determination.

In mouse, *Sox9* expression was maintained in the testis during fetal and adult life, but no expression was detected in ovary at any developmental stages (Kent *et al.*, 1996). The gene knock-out experiment also proved that *Sox9* is essential to mediate a switch from the ovarian pathway to the testicular pathway (Chaboissier *et al.*, 2004). In chickens, *Sox9* expression was first detected in the male genital ridge in 6.5 and 7.5 day embryos, but remained absent in the female genital ridge (Kent *et al.*, 1996). These results indicated that *Sox9* plays important roles

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Received: 11 January 2013 Accepted: 11 June 2014

in the male gonadal development, implying a role in sex determination and differentiation.

To date, studies have shown that *Sox9* is involved in gonadal differentiation in mammals as well as in reptiles (Modi and Crews, 2005; Western *et al.*, 1999). Consistent with the mammalian model, *Sox9* expression was upregulated in male embryonic gonad of certain reptiles with temperature-dependent sex determination (TSD) (Koopman, 2005; Shoemaker *et al.*, 2007; Western *et al.*, 1999). Unexpectedly, its expression was upregulated in whole embryo tissue at female- rather than male-producing temperatures in the TSD leopard gecko *Eublepharis macularius* (Valleley *et al.*, 2001).

Eremias multiocellata is a viviparous lizard which has been extensively explored in recent years (Li *et al.*, 2011; Tang *et al.*, 2013; Yue *et al.*, 2012). Our previous studies showed that *E. multiocellata* is a TSD species, skewing in favour of males at higher temperature (Tang *et al.*, 2012; Zhang *et al.*, 2010). Meanwhile, we have proven that this lizard may exhibit female heterogametic (ZZ/ZW) sex chromosomes by comparative genomic hybridization (unpublished). Therefore, it is possible that *E. multiocellata* is a transition species between TSD and genotypic sex determination (GSD). We previously performed a partial characterization of the *Sox* gene family of the *E. multiocellata*, and obtained short fragments of six different genes (Xin *et al.*, 2012). The mechanism of TSD in this species is still unclear and *Sox9* is an essential component of the testis-determining pathway that is conserved in species with TSD. In the present study, we 1) cloned and sequenced *Sox9* gene in this species and compared to other reptiles; and 2) analyzed its expression profile in different tissues of both male and female adult lizards.

2. Materials and methods

2.1 Animals and sampling *E. multiocellata* were captured in Minqin (38°38' N, 103°05' E), Gansu Province, China. The sex was identified by hemipene eversion. All experiments were approved by the Ethics Committee of Animal Experiments at Lanzhou University and in accordance with guidelines of the China Council on Animal Care.

For the extraction of RNA, the brain, heart, liver, kidney, gonads and muscle tissues were collected from five male and female adults, respectively. These tissues were rapidly put into liquid nitrogen, and stored at –80°C.

2.2 Total RNA extraction and cDNA synthesis Total RNA was isolated from *E. multiocellata* tissues using RNAiso Plus reagent (Takara, Dalian, China) according to the manufacturer's instruction. The total RNA concentration and integrity were determined using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and agarose-gel electrophoresis, respectively. Reverse transcription was carried out with 1μg total RNA using the PrimeScript® RT reagent Kit with gDNA Eraser (Takara, Dalian, China) to avoid genomic DNA contamination.

2.3 Molecular cloning of cDNA fragments Degenerate primers were designed using the CODEHOP tools (http://blocks.fhcrc.org/codehop.html) according to the multiple alignments of *Sox9* sequences in reptiles obtained by NCBI data. Primers F1R1 and F2R2 were used to amplify the partial fragments of *Sox9* (Table 1). Touch down PCR was performed in a Bio-Rad mastercycler gradient thermal cycler using Taq DNA polymerase (Sangon, Shanghai, China). The PCR product was gel purified, ligated into the pUCm-T vector, transformed into *Escherichia coli* DH5α competent cells and sequenced (Sangon, Shanghai, China). The identity of the obtained sequence was confirmed using BLAST analysis.

2.4 RACE analysis and cloning the full-length *Sox9* cDNA The RACE method was performed by using the SMART™ PowerScript Reverse Transcriptase (Clontech Laboratories) as described by the manufacture. Gene-specific primers for RACE were designed according to the previous isolated partial *Sox9* fragment. *Sox9*-GSP3 and *Sox9*-GSP4 primers were used in the first and second 3' RACE PCR, respectively. Subsequent cloning of RACE products were performed as described above.

2.5 Analyses of cDNA and its deduced amino acid sequence Deduced protein sequence was determined using the Expert Protein Analysis System translate program (http://web.expasy.org/translate/). The theoretical protein molecular weight and isoelectric point were obtained using the ProtParam tool program (http://web.expasy.org/compute_pi/). Transmembrane domain and N-terminal signal peptide were predicted by using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) and SignalP (http://www.cbs.dtu.dk/services/SignalP/), respectively. A phylogenetic tree of different vertebrate Sox9 proteins was constructed using neighbor-joining (NJ) and maximum parsimony (MP) methods with the software of Clustal X2 and MEGA4.0 (Tamura *et al.*, 2007). Sequences used for alignments/phylogenetic trees and their GenBank accession numbers were listed in Table 2.

2.6 Expression analysis by real-time RT-PCR The *Sox9* mRNA expression level in different tissues was quantified by using SYBR Green I detection method. Total RNA extraction, DNase I treatment and cDNA synthesis were performed as described above. Specific primers *Sox9*-F3R3 and β-actin F2R2 (Table 1) were used for gene expression analysis. β-actin served as an internal control. Real-time quantitative PCR (qPCR) was performed in a 20 μl reaction volume that contained 2 μl cDNA template, 10 μl 2×SYBR Premix Ex Taq II and 0.2 μM of each primer (Takara, Dalian, China).

The expression level of genes could be calculated by $2^{-\Delta\Delta CT}$ and three repeats were performed for each sample. The statistical analyses with one-way ANOVA were performed by SPSS 20.0 software.

3. Results

3.1 Molecular characteristic of *E. multiocellata Sox9* Based on the results of sequence analysis obtained from several RACE runs and the touchdown PCR, a 2124 bp *Sox9* cDNA sequence of *E. multiocellata* was isolated, including an open reading frame (ORF) and 3' untranslated region. Although we failed to isolate the 5' untranslated region, another primer GSP1 started with ATG was designed and used in combination with GSP2 to obtain the whole 1497 bp open reading frame. In the 3' RACE, a 627 bp product was identified including a polyadenylation signal (AATAA) at nucleotide positions 1799–1824, upstream from the poly (A) tail.

The ORF sequence of *EmSox9* encodes a protein of 498 amino acids with a predicted molecular weight of 55.45 kDa and isoelectric point of 6.31. No hydrophobic transmembrane region and signal peptide cleavage site were found in the predicted structure of the *EmSox9*. This predicted protein contains a characteristic HMG-box DNA-binding domain of 79 amino acids, which is located at 103–181. The nucleotide and deduced amino acid sequence of *EmSox9* cDNA were shown in Figure 1. The sequence has been submitted to the GenBank database with the accession number KF700266.

The BLAST analysis revealed that *EmSox9* shares high homology to the *Sox9* of other species. In general, *EmSox9* showed an overall amino acid identity of >82% compared to other reptiles (Table 2). The high sequence homology for *Sox9* from different species indicated the conservation of the Sox9 protein throughout the long term of evolution (Figure 2).

3.2 Phylogenetic relationship of *Sox9* Phylogenetic tree was constructed by the NJ method based on the amino acid sequences of many species (Figure 3). The NJ-trees revealed that *EmSox9* clustered with that of other reptiles. MP tree also showed the similar topology.

3.3 Expression of *Sox9* in adult tissues The relative levels of the *Sox9* mRNA in different tissues and sexes were determined by qPCR with *Sox9*-F3R3 primer (Figure 4). Each sample was normalized with β-actin. The result revealed that *Sox9* was ubiquitously expressed in brain, heart, liver, kidney, muscle, testis and ovary with most abundant transcripts in brain and testis. The expression of *Sox9* in testis was significantly increased than in ovary ($P < 0.01$). Exceedingly low levels of *Sox9* transcripts were detected in muscle and ovary. It is worth noting that the expression level of *Sox9* was higher in female than in male except for gonadal tissue, showing a clear tissue-specific pattern of sexual dimorphism.

4. Discussion

In the present study, we cloned the *Sox9* gene in *E. multiocellata*. A fragment of 621 bp of *Sox9* was amplified using the degenerate primers at first. Then, an ORF which encodes a protein of 498 amino acids and a 626 bp of 3'-untranslated region was obtained by the RACE method. Amino acid sequence of *EmSox9* has almost identical domain aligned with the orthologues of other vertebrates. The HMG-box of *EmSox9* was located at 103–181aa and no amino acid change was found compared to reptiles and mammals (Figure 2). At either end of the *Sox*-HMG domain there are consensus nuclear localization signal (NLS) sequences which may interact with some nuclear transport proteins (Harley, 2002). Both NLS motifs are conserved in many HMG domain proteins, suggesting that this mechanism of nuclear localization is common to the Sox family (Wilson and Koopman, 2002). In addition, we also observed a conserved pair of C-terminal trans-activation domains in *EmSox9*, comprising proline-glutamine-alanine (PQA) and proline-glutamine-serine (PQS). Sequence lengths of these fragments around the PQA-rich region displayed high variability, which causes the differences in the length of Sox9 proteins among different species (Figure 2). Several studies indicated that PQA and PQS rich regions of *Sox9* are related to maximal transcriptional activation, and mutations truncated the C-terminal transactivation domain might influence the ability of *Sox9* to activate target genes during organ development (Sudbeck *et al.*, 1996). More recently, a dimerization domain has been identified at the N-terminus, which is involved in DNA-dependent dimerization of *Sox9* to control chondrocyte

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1      ATGAATCTCCTGGACCCTTTCCTCAAGATGACCGAGGAGCAGGAGAAATGCCTGTCCGGC
1      M  N  L  L  D  P  F  L  K  M  T  E  E  Q  E  K  C  L  S  G
61     GCCCCCAGCCCCACCATGTCGGACGACTCGGCCGGCTCCCCTTGCCCTTCGGGCTCTGGA
21     A  P  S  P  T  M  S  D  D  S  A  G  S  P  C  P  S  G  S  G
121    TCGGACACCGAGAACACGCGTCCCCAAGAGAACACGTTCCCCAAAAGCGACCCGGACCTG
41     S  D  T  E  N  T  R  P  Q  E  N  T  F  P  K  S  D  P  D  L
181    AAGAAAGAGAGCGACGAGGACAAGTTCCCCGTGTGCATCCGAGAAGCCGTCAGCCAGGTC
61     K  K  E  S  D  E  D  K  F  P  V  C  I  R  E  A  V  S  Q  V
241    CTGAAGGGCTACGACTGGACGCTCGTGCCCATGCCCGTGCGCGTGAACGGATCCAGCAAG
81     L  K  G  Y  D  W  T  L  V  P  M  P  V  R  V  N  G  S  S  K
301    AACAAGCCCCACGTCAAGCGCCCCATGAACGCCTTCATGGTGTGGGCGCAGGCCGCGCGC
101    N  K  P  H  V  K  R  P  M  N  A  F  M  V  W  A  Q  A  A  R
361    AGGAAGCTCGCCGACCAGTATCCGCACCTGCACAACGCCGAGCTGAGCAAGACGCTGGGC
121    R  K  L  A  D  Q  Y  P  H  L  H  N  A  E  L  S  K  T  L  G
421    AAACTCTGGAGGTTATTGAATGAGAGCGAGAAACGCCCATTTGTGGAGGAGGCGGAAAGG
141    K  L  W  R  L  L  N  E  S  E  K  R  P  F  V  E  E  A  E  R
481    CTGAGGGTGCAGCATAAAAAAGACCATCCAGACTATAAGTACCAGCCACGAAGGAGAAAG
161    L  R  V  Q  H  K  K  D  H  P  D  Y  K  Y  Q  P  R  R  R  K
541    TCGGTCAAGAATGGCCAGGCTGAGCAAGAGGAAGGATCTGAGCAAACTCACATCTCCCCT
181    S  V  K  N  G  Q  A  E  Q  E  E  G  S  E  Q  T  H  I  S  P
601    AATGCCATCTTCAAGGCCCTGCAGGCAGATTCTCCCCAGTCTTCATCCAGCATGAGCGAA
201    N  A  I  F  K  A  L  Q  A  D  S  P  Q  S  S  S  S  M  S  E
661    GTGCATTCCCCTGGGGAACATTCTGGACAGTCTCAAGGGCCACCAACCCCTCCCACTACC
221    V  H  S  P  G  E  H  S  G  Q  S  Q  G  P  P  T  P  P  T  T
721    CCTAAAACAGATGTGCAGCCTGGCAAGCAGGACCTGAAAAGGGAAGGGCGCCCCCTGCAG
241    P  K  T  D  V  Q  P  G  K  Q  D  L  K  R  E  G  R  P  L  G
781    GAGGGAGGGAGGCAGCCGCCCCACATTGACTTCCGAGATGTGGACATTGGTGAGCTCAGC
261    E  G  G  R  Q  P  P  H  I  D  F  R  D  V  D  I  G  E  L  S
841    AGCGATGTCATCTCCAACATCGAGACCTTTGATGTAAACGAGTTTGACCAGTATCTCCCA
281    S  D  V  I  S  N  I  E  T  F  D  V  N  E  F  D  Q  Y  L  P
901    CCCAACGGCCACCCTGGCGTCCCGGTTACCCATGGCCAGCCTGGCCAAGTCACCTATACC
301    P  N  G  H  P  G  V  P  V  T  H  G  Q  P  G  Q  V  T  Y  T
961    GGCAGCTACGGAATCAGCAGCACGGCAGCCGCTCCAGCCGGCACAGGGCATGTCTGGATG
321    G  S  Y  G  I  S  S  T  A  A  A  P  A  G  T  G  H  V  W  M
1021   TCCAAGCAGCCGCAGCAGCCGCCACAGCCGCCTCAGCAGCCACCGTCCCAAGCGCCGCAG
341    S  K  Q  P  Q  Q  P  P  Q  P  P  Q  Q  P  P  S  Q  A  P  Q
1081   CAGGCCCCGCAGCCGCCACAGCACACCTTAACCACGCTGAGCAGCGAGCCA
361    Q  A  P  Q  P  P  Q  H  T  L  T  T  L  S  S  E  P  G  Q  A
1141   CAGCAGAGGACACACATCAAGACGGAACAGCTGAGCCCCAGCCATTACAGC
381    Q  Q  R  T  H  I  K  T  E  Q  L  S  P  S  H  Y  S  E  Q  Q
1201   CAACACTCGCCCCAGCAGATCAGCTACAGCTCCTTCAACCTGCAGCACTACAGCTCCTCC
401    Q  H  S  P  Q  Q  I  S  Y  S  S  F  N  L  Q  H  Y  S  S  S
1261   TACCCCACCATCACTCGCTCGCAGTACGACTACACAGACCACCAGAGCTCCAACTCCTAC
421    Y  P  T  I  T  R  S  Q  Y  D  Y  T  D  H  Q  S  S  N  S  Y
1321   TATAGCCATGCTGCTGGCCAGAGCTCCAGCCTCTACTCGACCTTCACGTACATGAACCCT
441    Y  S  H  A  A  G  Q  S  S  S  L  Y  S  T  F  T  Y  M  N  P
1381   GCCCAGAGGCCGATGTACACGCCCATTGCAGACACCGCCGGGGTGCCTTCCATTCCTCAG
461    A  Q  R  P  M  Y  T  P  I  A  D  T  A  G  V  P  S  I  P  Q
1441   ACCCACAGCCCGCAACACTGGGAGCAGCCTGTGTATACACAGCTCACCAGGCCATAA
481    T  H  S  P  Q  H  W  E  Q  P  V  Y  T  Q  L  T  R  P  *
       GGTTTTTGACAGACAGCAAAGTGTCTACAGACTTTGGTGAAAGTTTCTTTTTTGAAGAAG
       AAGGAGGAGGAGAAGGAGAAGGAGGAGAAGGAGGAGAAGAAAGCTAAACATGTACAACTCG
       AGAGACAGGAAAATCCCAGAATGCCTTGTGTGCTACAGCATCCTTTTGAAATCAGCCAGA
       GAATTCTGCTCCATAAACAAGCATTCCATGGTGGACTTAGAACTTTGCTTTGGAGGCGAT
       TTTCATTCCTAGACAGCCAGCTTGGTTCACCTAGGTATGGACTTGGTGATTATTTTTTAA
       CAATAACTAAAAATGAAAAGCGAATAAATTTTTTATTATTATTACAAAACTCAAAAGTATT
       CCTCTGTGAGGAATATGCTCTATTCTAAGTATTTTGTTTTGTTTTAGTATGTACTG
       TGTATGATTCATTACCAATTTGAGGGGCTTTTATATGGAATTGTACTTCTTGTTTTTAGA
       GAAACTGGTTTTGTTTTAAAATGCTCCTTATTTTGACAGCTAAACAACACTTAGCAAACA
       CGGCATGCCTTAGCCAGAGGTATTTTTGTATAGTTAATGGTCTTTCGCTTATAGGCAGCA
       AAAAATTGTGCATAAAAGAAAAGCAAA
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Figure 1 Nucleotide sequence and deduced amino acid sequence of the *E. multiocellata Sox9* cDNA. Italicised numbers indicate the nucleotide position starting at the ATG start codon. The HMG domain is indicated by a box. A putative poly (A) signal, AATAA, is underlined.

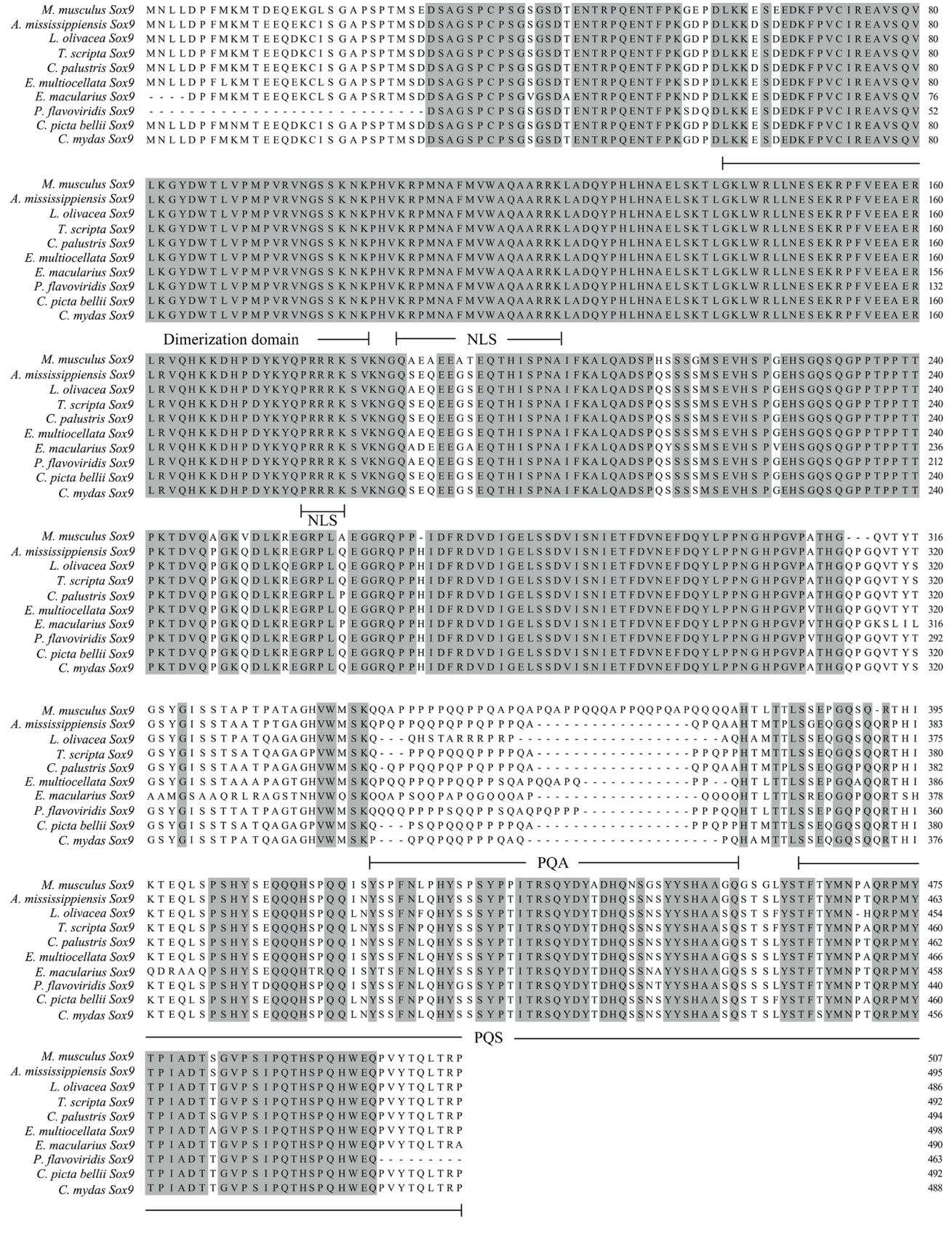

Figure 2 Multiple alignments of amino acid sequences of *Sox9* from *E. multiocellata* and other species. Conserved sites of Sox9 proteins are shaded. The functional domains dimerization domain, nuclear localization signal (NLS), proline-glutamine-alanine (PQA), proline-glutamine-serine (PQS) are underlined.

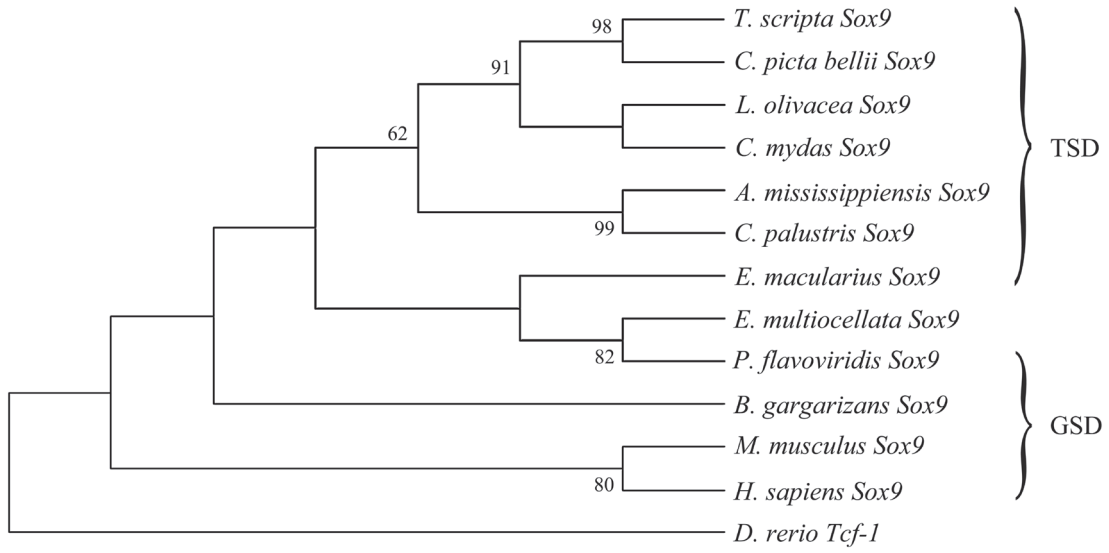

Figure 3 A phylogenetic tree produced by the neighbour joining method that illustrates the relationship between Sox9 proteins from different species. Bootstrap values based on 1000 replicates. The scale bar indicates the branch length. The outgroup was provided by the distantly-related HMG-box protein TCF-1 from *Danio rerio* (GenBank accession number CAB59622.1).

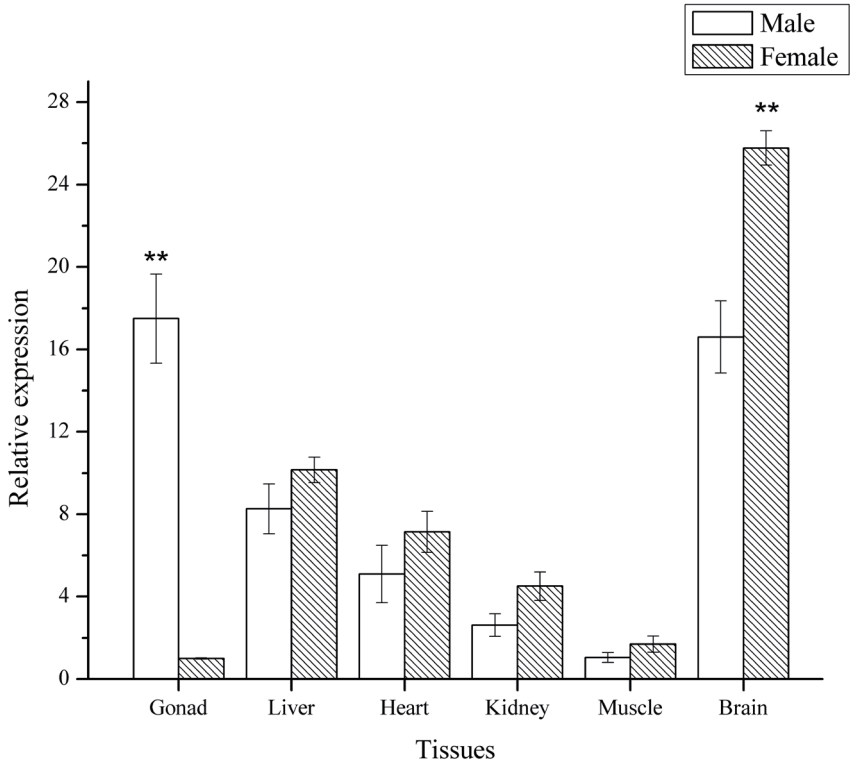

Figure 4 Relative expression levels of *E. multiocellata Sox9* gene in various tissues. Data were presented as mean (n = 3) ± SE, means were compared by one-way ANOVA and Tukey's multiple comparisons test ($P < 0.05$).

differentiation, but not for sex determination (Bernard *et al.*, 2003; Sock *et al.*, 2003). Only one amino acid mutation (Glu → Asp) in the dimerization domain of *EmSox9* is found, which appears to be unique for reptiles.

Reptiles exhibit extraordinary variability in sex chromosome structure and patterns of sex determination among vertebrates (Valenzuela and Lance, 2004). Although the triggers to initiate sex determination may vary greatly, there is considerable conservation in the genetic cascades including the gene *Sox9* involved in sex

Table 1 List of primers used for PCR amplification.

Primer Name	Sequence (5'–3')
Sox9-F1	TCGAGACCTTCGACGTGAAYGARTTYGA
Sox9-R1	TGGGTGTACACGGGCTGYTCCCARTGYTG
Sox9-F2	TACGACTGGACCCTGGTGCCCATG
Sox9-R2	TCCGTCTTGATGTGTGTCCTCT
Sox9-GSP1	ATGAATCTCCTGGACCCTTTCCTCAAGA
Sox9-GSP2	GAA TCT GCC TGC AGG GCC TTG AAG ATG
Sox9-GSP3	TCACTCGCTCGCAGTACGACTACAC
Sox9-GSP4	GCCTTCCATTCCTCAGACCCACAG
Sox9-F3	GAGGACACACATCAAGACGGAAC
Sox9-R3	AGTGATGGTGGGGTAGGAGGA
β-actin F1	TGATGGTGGGCATGGGNCARAARGA
β-actin R1	CACGGCCTGGATGGCNRCRTACAT
β-actin F2	CCCATTGAGCACGGCATT
β-actin R2	CTTTTCCCTGTTGGCTTTGG

Table 2 Percent identities of amino acid sequences of *Sox9* between *E. multiocellata* and other species.

Species Name	Accession Number	Identities (%)
Mus musculus	NP_035578.3	87
Homo sapiens	NP_000337.1	87
Bufo gargarizans	ABI34568.1	85
Alligator mississippiensis	AAD17974.1	93
Trachemys scripta	ACG70782.1	92
Lepidochelys olivacea	ACT82009.1	91
Chelonia mydas	EMP39318.1	91
Chrysemys picta bellii	XP_005283023.1	92
Crocodylus palustris	ACU12296.1	93
Protobothrops flavoviridis	BAF36483.1	93
Eublepharis macularius	AAG36779.1	88
Anolis carolinensis	XP_003224717.1	82

determination across vertebrates (Crews, 2003). In this study, the phylogenetic tree of *Sox9* appears to group five clusters. It is in close accord with the established taxonomic relationship and reflects the shift of sex determination modes successively from TSD to GSD (Figure 3). *EmSox9* displays the highest similarity to those of turtles and crocodiles which prove to be TSD species (Ferguson and Joanen, 1982; Lang *et al.*, 1989; Merchant-Larios *et al.*, 1997; Morjan, 2003; Spotila *et al.*, 1987; Wibbels *et al.*, 1998), in accord with previous study that *Sox9* plays conservative roles in male development of TSD species. Meanwhile, the *EmSox9* closely clustered with the *Sox9* of snake *P. flavoviridis* having ZZ/ZW sex determination system (Kawai *et al.*, 2009) rather than *E. macularius* exhibiting TSD (Valleley *et al.*, 2001). Our previous study also found that *E. multiocellata* exhibits both TSD and ZZ/ZW sex determination (Tang *et al.*, 2012; Zhang *et al.*, 2010). Thus, the phylogenetic tree for *EmSox9* provides indirect evidence that *E. multiocellata* may be a transition species between TSD and GSD.

Sox9 is a member of group E of *Sox* genes (Bowles *et al.*, 2000), known to be important for the process of sex determination in vertebrates (Koopman, 2005). The present study showed that *Sox9* is widely expressed in *E. multiocellata* adult tissues, with highest levels in testis and brains. Moreover, the expression of the *Sox9* was higher in female than in male except for gonad tissue. To our knowledge, this tissue-specific and sexual dimorphic expression of *Sox9* has been found ubiquitous in a great many species.

The extensive expression of *Sox9* gene in the brain has also been observed in other species, such as *Rana rugosa*, *Clarias gariepinus* and *Crocodylus palustris* (Agrawal *et al.*, 2009; Raghuveer and Senthilkumaran, 2010; Takase *et al.*, 2000). These facts suggest that *Sox9* expression in brain tissue is conserved, and *Sox9* may be a central element in neurogenesis and chondrogenesis of vertebrates. The possible role of *Sox9* in brain development in *E. multiocellata* needs to be further investigated.

EmSox9 was highly expressed in the adult brain and testis, but weakly expressed in the ovary. This pattern

indicates that *Sox9* may be critical for final commitment to a testicular fate in *E. multiocellata*. This assumption has been supported by several researches. *Sox9* is predominantly expressed in developing male gonads of chicken (Kent *et al.*, 1996), turtle and alligator (Torres Maldonado *et al.*, 2002; Western *et al.*, 1999) which have different sex determination mechanisms. The mechanisms of sex determination are rather diversified among vertebrates. In the same way, the function of *Sox9* in sex determination may be complicated, further research is needed to elucidate the precise role and mechanism in sex determination of *Sox9* in *E. multiocellata.*

Acknowledgements

We are grateful to M. Ma, J.R. Mo for help in various ways. This work was supported by the National Natural Science Foundation of China (No. 30670263).

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